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Abstract: Suitable nutrition at first feeding in marine fish larvae is an important factor for successful larval and juvenile rearing. In this way probiotic supplementation may contribute to better face the habitual stress of metamorphosis and weaning at early sole stages. The aim of this study was to optimize S. putrefaciens Pdp11 administration to Senegalese sole (Solea senegalensis) at early stages of development. For this purpose, we studied the influence of a short pulse of Shewanella putrefaciens Pdp11 (10-30 dah) on growth performance, gut microbiota modulation, digestive enzymes activities and body composition. These probiotic bacteria were incorporated using Artemia as live vector (2.5 ? 107cfu mL-1) and supplied to sole specimens in a co-feeding regime (Gemma, Skretting) by triplicate. S. putrefaciens Pdp11 colonized larval gut being present from 23 to 119 dah. Probiotic supply significantly modulated larval and fry gut microbiota. A PCA analysis including all the parameters analyzed strongly associated S. putrefaciens Pdp11 presence with a higher fish growth, a higher digestive proteolytic activity level and a fish body composition modulation along S. senegalensis rearing. In addition less size variability was obtained from metamorphosis until the end of weaning. In conclusion a short pulse of S. putrefaciens Pdp11 (10-30 dah) seems sufficient to obtain a suitable microbial modulation involved in a better growth performance and body composition that suggests a great potential for sole aquaculture production.

Highlights

- A short pulse of *Shewanella putrefaciens* Pdp11 probiotic addition (10-30 dah) is sufficient to obtain a suitable microbial modulation in *Solea senegalensis* larviculture.
- The microbial modulation obtained was linked to a digestive enzyme stimulation.
- Both enhanced parameters involved a better growth performance and body composition in sole first stages of culture.

Benefits of probiotic administration on growth and performance along metamorphosis and weaning of Senegalese sole (*Solea senegalensis*)

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Abstract

Suitable nutrition at first feeding in marine fish larvae is an important factor for successful larval and juvenile rearing. In this way probiotic supplementation may contribute to better face the habitual stress of metamorphosis and weaning at early sole stages. The aim of this study was to optimize *S. putrefaciens* Pdp11 administration to Senegalese sole (*Solea senegalensis*) at early stages of development. For this purpose, we studied the influence of a short pulse of *Shewanella putrefaciens* Pdp11 (10-30 dah) on growth performance, gut microbiota modulation, digestive enzymes activities and body composition. These probiotic bacteria were incorporated using *Artemia* as live vector (2.5×10^7cfu mL^{-1}) and supplied to sole specimens in a co-feeding regime (Gemma,

Skretting) by triplicate. *S. putrefaciens* Pdp11 colonized larval gut being present from 23 to 119 dah. Probiotic supply significantly modulated larval and fry gut microbiota. A PCA analysis including all the parameters analyzed strongly associated *S. putrefaciens* Pdp11 presence with a higher fish growth, a higher digestive proteolytic activity level and a fish body composition modulation along *S. senegalensis* rearing. In addition less size variability was obtained from metamorphosis until the end of weaning. In conclusion a short pulse of *S. putrefaciens* Pdp11 (10-30 dah) seems sufficient to obtain a suitable microbial modulation involved in a better growth performance and body composition that suggests a great potential for sole aquaculture production.

Keywords: body composition, digestive enzymes, growth, gut microbiota, larviculture, probiotics, *Shewanella*, *Solea senegalensis*.

1. Introduction

Senegalese sole (*Solea senegalensis*) is a promising flatfish species for intensive marine farming (Conceicao et al., 2007) due especially to its high market value (Borges et al., 2009). Despite recent significant advances in weaning techniques and larvae feeding (Imsland et al., 2003; Engrola et al., 2007), the production of high quality sole juveniles is still a bottleneck (Dámaso-Rodrigues et al., 2010). The main factors affecting *S. senegalensis* production performance are disease control, advanced weaning, nutrient requirements and optimization of feeding protocols (Conceicao et al., 2007; Engrola et al., 2007). In addition, the high amplitude of size dispersion coefficients in *S. senegalensis* cultivated stocks is still an important issue in the sole farming industry (Salas-Leiton et al., 2010; Sánchez et al., 2010).

Appropriate nutrition at first feeding in marine fish larvae including *Solea solea* and *S. senegalensis* is an important factor for successful larval and juvenile rearing (Heath and Moore, 1997; Dámaso-Rodrigues et al., 2010). Marine fish larvae have generally a poorer capacity to digest and/or absorb complex nutrients in comparison with larger fish (Ronnestad and Conceicao, 2005), and much higher growth rates (Conceicao et al., 1998). This very high growth potential of fish larvae means greater requirements in terms of energy, amino acids (AAs), highly unsaturated fatty acids (HUFAs), phospholipids (PLs) and other nutrients. However the exact requirements for fish larvae are poorly characterized, even for the better studied species (Conceicao et al., 2007; Rodríguez et al., 2009). The protein and lipid requirements of fish have been reported to vary according to many

different factors such as the species size or age, dietary protein quality and level of energy, water quality, presence of natural food and feeding and culture management (NCR, 1993; Rodríguez et al., 2009), even though it is well known that lipid/protein ratio influences fish growth and nutrient efficiency (Sargent et al., 2002; Ronnestad and Conceicao, 2005; Aliyu-Paiko et al., 2010; Li et al., 2012). In this way a suitable balance of lipids can help the fish achieve the so called protein sparing effect, by means of a shift of dietary protein from energetic purposes into growth (Ronnestad and Conceicao, 2005; Li et al., 2012) Taking into account these last considerations, digestion is a particularly relevant process in animal nutrition since it influences the bioavailability of nutrients needed for fish growth. The analysis of digestive enzymes is a key tool when studying the nutritional condition and adaptation of fish to dietary changes. In particular, the evolution of digestive activities during larval maturation of S. *senegalensis* has twofold interest. On the one hand, the assessment of the presence and level of certain enzymatic activities may be used as a comparative indicator of the fish development rate, as well as of their further survival rate (Cara et al., 2007). On the other hand, changes in enzymatic activities can be used for studying the effects of the dietary additives that might modulate the maturation process of the digestive system (Gisbert et al., 2009). In this way dietary probiotic addition is being increasingly reported as an enzymatic contributor to digestion (Tinh et al., 2008; Sun et al., 2013).

Fish larvae are exposed to microbiota-associated disorders because they start feeding when the digestive tract is not fully developed (Sttotrup and McEvoy, 2003; Ronnestad and Conceicao, 2005) and the immune system is still incomplete (Vadstein, 1997). For this reason probiotic treatments are particularly desirable at these stages (Tinh et al., 2008) providing a balanced gut microbiota condition (Olafsen, 2001).

The gastrointestinal tract serves as a route for entry of pathogenic microorganisms (Ringo et al., 2004; Chen et al., 2008) and it is believed to be the major route for the onset of diseases like vibriosis, furunculosis, enteric septicaemia and aeromoniasis in fish (Nayak et al., 2010). A healthy intestinal microbiota not only aids the digestive function but also acts by inhibiting pathogens (Sugita et al., 2002; Makridis et al., 2005). In a healthy specimen a proper balance between the intestinal microbiota and the host's control mechanism (Sansonetti et al., 2004; Nayak et al., 2010) occurs and if this balance is disturbed, the pathogens can establish infections (Virgin, 2007; Sekirov and Finlay, 2009).

In addition probiotic application has increased in fish aquaculture based on the beneficial effects obtained previously in livestock (Fulton et al., 2002) and humans (Gills, 2003). Due to the different environmental conditions of aquatic animals, probiotics have frequently been selected from specimens and environmental autochthonous bacteria (Chabrillón et al., 2005a,b; Lauzon et al., 2010). In this way *Shewanella putrefaciens* Pdp11, isolated from the skin mucus of healthy cultured gilthead seabream *Sparus aurata*, was *in vitro* selected by its antagonism against the main pathogens of *S. senegalensis* (Chabrillón et al., 2005a). This probiotic strain has increased *in vivo* pathogen resistance of *S. senegalensis* (Díaz-Rosales et al., 2009; García de la Banda et al., 2010, 2011; Tapia-Paniagua et al., 2012), and *S. aurata* on-growing (Salinas et al., 2006; Varela et al., 2010). Furthermore *S. putrefaciens* Pdp11 enhanced growth, improved biochemical composition, activated digestive enzyme activity and improved intestinal epithelium integrity in sole juveniles (Sáenz de Rodrigáñez et al., 2009; García de la Banda et al., 2010, 2012). Moreover it has been reported that *S. putrefaciens* Pdp11 colonized sole gut modulating intestinal microbiota (Tapia-Paniagua et al., 2012; Lobo et al., 2013).

In a previous larval trial it was demonstrated that continuous administration of *S. putrefaciens* Pdp11 bioencapsulated in *Artemia* until the end of weaning period modulated gut microbiota increasing growth performance and feed utilization efficiency in *S. senegalensis* fry (Lobo et al., 2013). In addition (García de la Banda et al., 2011) evidence of a rapid immune stimulation in sole juveniles was apparent by *S. putrefaciens* Pdp11 supply in feed. Considering the above mentioned, the aim of the present study was to assess the effect of a short pulse of probiotic during metamorphosis (from 10 to 30 dah) on growth performance, gut microbiota modulation, digestive enzymes and body composition in *S. senegalensis* at early development.

2. Material and Methods

2.1. *Microorganisms*

Shewanella putrefaciens Pdp11 was grown in 5 mL of tryptone soya broth (Oxoid Ltd., Basingstoke, UK) supplemented with 1.5% NaCl (TSBs) for 18 h at 22ºC, with continuous shaking. Aliquots of 0.1 mL of the culture were spread onto plates of tryptone soy agar, (Difco™, Sparks, USA), supplemented with 1.5% NaCl (TSAs) and incubated daily at 22ºC. Bacterial suspensions were prepared by scraping the cells from the plates and suspending them in sterile phosphate-

buffered saline (PBS, pH 7. The number of bacterial cells mL^{-1} was measured at 600nm by using a Hach DR/2500 Laboratory Spectrophotometer (Loveland, Colorado, USA). Probiotic cells were supplied (2.5×10^7cfu mL^{-1}) at the final period of enrichment of Artemia (200-300 metanauplii mL^{-1}). This dose has been previously reported as suitable by Lobo et al. (2014) and is in the range of other probiotics used in larviculture (Dias et al., 2004; Hernández-Martínez et al., 2009). No probiotic cells were supplied to an *Artemia* incubator used as control. After an incubation period of 3 - 9 h the concentration of total bacteria was 10^4 cfu *Artemia* metanaupli^{-1} where *S. putrefaciens* Pdp11 cells accounted at least for 50%. These values are similar to those reported by other authors (Villamil et al., 2003; Carnevali et al., 2004). No mortalities of *S. putrefaciens* Pdp11 nor of *Artemia* metanauplii were registered during the incubation period. Artemia from both incubators (45 L) were maintained at 23ºC and rinsed with 1 µm filtered seawater for five minutes prior to their supply to rearing tanks.

S. putrefaciens Pdp11 was characterised by using ID-GNB Vitek 2 (Biomerieux, Marcy L'Etoile, rance) including 64 tests. Probiotic strain was cultivated on TSAs at 22ºC for 2 days and pure colonies suspended in sterile PBS (pH 7.2). The strip was inoculated and incubated at 22ºC for 4 h. A rapid identification of constitutive enzymes based on colour development due to enzyme activity was then performed.

2.2. *Larval rearing conditions*

Embryos were incubated at 19.0 ± 0.5 ºC in 70 L cylinder-conical incubating tanks with gentle aeration and a continuous water flow of ten times per day. Newly hatched larvae (40 individuals L^{-1}) were randomly distributed into 250 L circular polyester tanks by triplicate, with a constant aeration and seawater renewal. Temperature varied between 17.8 ± 0.8 ºC (1-56 dah), 18.9 ± 0.4 ºC (57-85 dah) and 19.6 ± 1.1 ºC (85-117 dah). Salinity was 35.4 g L^{-1} throughout the trial. Illumination (1000 lux on surface water) was provided with Sylwania mini-lynx fast start lamps. It was continuous until 10 dah and a 12:12 L:D cycle was established until 21 dah. Afterwards postlarvae were reared in semidarknesss (< 20 lux on surface). Continuous water inflow was maintained to provide suitable oxygen and nitrite seawater levels for larval and postlarval culture (Parra and Yúfera, 1999; Lund et al., 2007). Once the larvae metamorphosed and became benthic, the experimental tanks were emptied, and survival checked. Fish were then randomly redistributed relative to both dietary treatments assayed and stocked at a density of 3000 individuals m^{-2} (each

dietary treatment 3 replicates). In addition, due to the high stocking density, on 41 dah postlarvae from each replicate were taken out to adjust density to 2500 individuals m^{-2}. Finally, after the weaning period (on 87 dah), the tanks were emptied and fish were randomly redistributed and stocked by triplicate at a density of 1250 individuals m^{-2} until the end of the feeding trial.

The feeding regime was based on Cañavate and Fernández-Díaz (1999). From 3 to 9 dah *Isochrysis galbana* enriched rotifers were added to the tanks twice a day to maintain a rotifer density of 20 individuals mL^{-1}. Microalgae (*Nannochloropsis gaditana,* 3×10^5 cells mL^{-1} and *I. galbana*, 7×10^4 cells mL^{-1}) were also supplied during this period to ensure a good rotifer quality. From 10 to 57 dah co-feeding was carried out with *Artemia* and the commercial pellet Gemma Micro Diamond (crude protein 57% and total lipids 15%, Skretting, Burgos, Spain). *Artemia* nauplii (AF strain INVE Aquaculture, Ghent, Belgium), was supplied from 10 to 12 dah and *Artemia* metanauplii (EG strain INVE Aquaculture, Ghent, Belgium) thereafter. Both *Artemia* stages were previously enriched with Origreen (fresh protein 43% and total lipids 30% Skretting, Burgos, Spain) for eighteen hours, and then supplied to the tanks four times a day, whereas dry feed was supplied eight times a day. Weaning started at 58 dah (Gemma Wean Diamond, crude protein 60% and total lipids 15%, Skretting, Burgos, Spain). Larvae were fed exclusively with dry feed (Gemma Diamond), after 87 dah. The amount of inert feed was gradually increased from 58 dah (39 g m^{-2}, 69.2 % of total feed) to 87 dah (117 g m^{-2}), while *Artemia* doses were progressively reduced from 14 metanauplii mL^{-1}. At the end of the trial, fries were fed 11.3% of total tank biomass. Two live feeding regimes were compared: Pdp11 and Control groups. Pdp11 group consisted of *S. putrefaciens* Pdp11 bacterial strain bioencapsulated in Origreen enriched *Artemia*. The treatment was given three times a day (from 10 to 30 dah), whereas no bacteria were administered to the Control group. After 30 dah and until the end of weaning all larvae were fed with *Artemia* control. Each dietary treatment was evaluated by triplicate.

2.3. *Growth and survival*

For growth studies, thirty specimens from each replicate were weekly and randomly sampled. Fish total length was measured by a profile projector (V-12B Nikon, Japan) until 60 dah, and by an ichthyometer until the end of the trial. After length measurement, specimens were rinsed with distilled water, put onto pre-weighed glass fiber filters, and dried at 60 ºC for 48 h, in order to obtain larval and fry dry weight. Survival was checked along the experiment

2.4. *Analysis of body composition*

Samples from Control *Artemia* and *S. putrefaciens* Pdp11 bioencapsulated *Artemia* metanauplii were collected in three eppendorf tubes, immediately frozen and stored at –80 ºC for the determination of total soluble protein and lipid contents. For the study of larval and fry body composition five samples were randomly collected from each rearing tank on days 23 (75), 56 (15), 87 (6) and 119 (6), the numbers in parenthesis being the total number of specimens sampled per replicate. Fish samples were washed several times with distilled water prior to being frozen at –80 ºC, until analysis. Total soluble protein was determined following the method of Bradford (1976). Total lipid content was assessed by extraction with chloroform-methanol 2:1 as described by Bligh & Dyer *(*1959) modified by Fernández-Reiriz *et al* (1989) and gravimetrically determined after centrifugation.

2.5. *Analysis of gut microbiota*

Six larvae from each batch were sampled on days 23, 56, 87 and 119 dah and analyzed for digestive microbiota. The whole intestines were aseptically removed and stored at –20 ºC until further analysis. The intestinal contents were homogenized in 1 mL PBS (pH 7.2), and 1 mL aliquot was centrifuged at 1000 × g for 5 min. Total DNA was extracted from samples as described by Martínez et al. (1998), with some modifications as described by Tapia-Paniagua *et al*. (2010). Agarose gel (1.5% [w/v]) electrophoresis in the presence of ethidium bromide was used to visually check for DNA quality and yield. To determine the presence of the probiotic *S. putrefaciens* Pdp11 in the intestinal microbiota of larvae, an axenic culture of the probiotic strain was grown up to the exponential phase in TSBs and then centrifuged at 2,500×g for 15 min. Pellets were washed with PBS and the resulting pellet was used for DNA extraction following the instructions of the Fast DNA Spin kit (Qbiogene, CA, USA).

In order to compare Denaturing Gradient Gel Electrophoresis (DGGE) patterns of the intestinal microbiota of soles receiving the different experimental treatments, the DNA was amplified using the 16S rDNA bacterial domain-specific primers 968-GC-F(5'CGCCCGGGGCGCGCCCCGGGCGGCC-GGGGGCACCGGGGGAACGCGAAGAACCTTAC-3') and 1401-R (5'CGGT-GTGTACAAGACCC-3') (Kostantinov et al., 2003; Kim and Austin, 2006) These primers were used to amplify V6-V8 regions of 16S rDNA,(55) and yields amplicons of 470-bp length. PCR

mixtures and conditions to perform PCR were those previously described by Tapia-Paniagua et al. (2010).

The amplicons obtained from the intestinal lumen-extracted DNA and the probiotic strain were separated by DGGE, according to the specifications of Muyzer et al. (1993) using a Dcode TM system (Bio-Rad Laboratories, Alcobendas, Spain). Electrophoresis was performed in an 8% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide; dimensions, 200 by 200 by 1 mm) using a 30 to 55% denaturing gradient for separation of PCR products. The gels contained a 30 to 55% gradient of urea and formamide increasing in the direction of the electrophoresis. A 100% denaturing solution contained 7 M urea and 40% (v/v) deionized formamide. PCR samples were applied to gels in aliquots of 13 µL per lane. The gels were electrophoresed for 16 h at 85 V in 0.5 X TAE (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2-EDTA) buffer at a constant temperature of 60 ºC (Sambrook et al., 1989) and subsequently stained with $AgNO_3$ (Sanguinetti et al., 1994). A DGGE analysis for all samples was performed twice.

DNA extracted from larvae fed the same diet and for a specific sampled time were mixed to carry out the cloning process. PCR was performed with a Taq DNA polymerase kit from Life Technologies. The forward primer S-D-Bact-0008-aS-20 (5' AGA GTT TGA TCC TGG CTC AG 3') (Hicks et al., 1992), which targets the domain Bacteria, and the reverse primer S-*-Univ-1492-b-A-21 (59ACG GCT ACC TTG TTA CGA CTT 3') (Kane et al., 1993) which amplifies the bacterial 16S rDNA. Amplification was carried out as described by Gray and Herwig (1996). Reaction tubes contained 100 ng of total DNA, 1.25 U of Taq DNA polymerase (from Life Technologies), buffer, 2.5 mM MgCl2, 200 µM dNTP´s each deoxyribonucleotide triphosphate, and 10 µM each primer in a final volume of 50 µL. Initial DNA denaturation and enzyme activation steps were performed at 94 °C for 10 min in a Eppendorf thermocycler, followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 48 °C for 1 min, and elongation at 72 °C for 1 min 30 s, which was followed by a final elongation at 72 °C for 5 min. PCR products were purified and concentrated with a High Pure Spin Kit PCR purification kit (Roche) according to the manufacturer instructions. Purifed PCR product was cloned into a pGEM-T (Promega, Madison, WI, USA). Ligation was performed at 4 ºC overnight followed by transformation into competent *E. coli* JM109. One hundred colonies of ampicillin-resistant transforms from each diet and day sampled (800 colonies) were transferred with a sterile toothpick to 100 µL TE buffer and boiled for 10 min at 95 ºC. PCR was then immediately performed with pGEMT- specific primers T7 (5'-AAT ACG ACT CAC TAT AGG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'). Plasmids containing

an insert were used to amplify the V6-V8 region of 16S rDNA. The amplicons were used for sequence analysis (Macrogen Korea sequencing). The resulting sequences (~500 bp) were compared with the sequences from the National Center for Biotechnology Information (NCBI) or Greengenes DNA sequence database using the BLAST sequence algorithm (Altschul et al., 1990).

2.6. *Intestinal enzyme analysis*

For the study of intestinal enzyme activities, five samples of feed larvae were randomly collected from rearing tanks, in triplicate (the numbers in parenthesis are the number of specimens sampled per replicate) at days 23 (75), 30 (30), 56 (3), 87 (3) and 119 (3). After being sampled, fish were rinsed with distilled water to remove external marine water, and then frozen with liquid nitrogen and stored at −80 ºC. For the preparation of enzymatic extracts, sampled larvae were individually dissected to remove head and tail under a binocular microscope on a pre-chilled glass plate maintained at 4 ºC. The visceral bulks were homogenized as pool (200 mg mL^{-1}) in cold distilled water. The supernatant obtained after centrifugation (16,000 x g for 15 min at 4 ºC) was stored at −20 ºC until enzymatic analysis. The concentration of soluble protein in aqueous extracts was determined by Bradford (1976) using bovine serum albumin (1mg mL^{-1}) as a standard.

Total alkaline protease activity was measured using azocasein (0.5%) in 100 mM Tris–HCl buffer, pH 9.0 as described Alarcón et al.(2007) Units of alkaline protease activity (UA) were calculated using the equation: $UA = \Delta Abs_{366nm} \times v_{reaction} \times time^{-1} \times v_{enzyme}^{-1}$, where t is min of enzymatic reaction, and $v_{reaction}$ and v_{enzyme} are expressed in mL. Trypsin activity was assayed using BAPNA (N-a-benzoyl-DL-arginine 4-nitroanilide hydrochloride) as substrate according to Erlanger et al. (1961). Chymotrypsin activity in extracts was determined using SAPNA (N-succinyl-ala-ala-pro-phe p-nitroanilide) according to Delmar et al. (1979). Leucine aminopeptidase was determined using leucine p-nitroanilide (0.1 mM in DMSO) as substrate, according to Maraux et al. (1973). For trypsin, chymotrypsin and leucine aminopeptidase activities one unit of enzyme activity was defined as 1 μmol p-nitroaniline released per minute using a coefficient of molar extinction of 8.8 at 410 nm. Determination of α-amylase activity was carried out using 2-chloro-p- nitrophenyl-α-D-maltotrioside as substrate, and rate of formation of the colored 2-chloro-p-nitrophenol was monitored kinetically at 405. One unit of enzyme activity was defined as 1 μmol of 2-chloro-p-nitrophenol released per minute using a coefficient of molar extinction of 12.9. Alkaline phosphatase activity was assayed using 4-nitrophenyl phosphate in 1 M diethanolamine, 1 mM

MgCl2 buffer (pH 9.8) according to Bergmeyer (1974). One unit was defined as 1 μg nitrophenyl released per minute using a coefficient of molar extinction of 18.5 at 405 nm. Except for total alkaline protease activity, the remaining enzymatic assays were adapted to microplate. All assays were performed by triplicate at 25 ºC. Digestive enzyme activities were expressed as U mg protein^{-1} and U larva^{-1} using the total number of larvae in each homogenized pooled sample.

Additionally, biochemical analysis of total alkaline protease was complemented with a study of intestinal protease isoforms. For this purpose, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of enzyme preparation from different larval ages was carried out in Mini Protean II chamber (Bio-Rad) according to Laemmli (1970) using 8 × 10 × 0.075-cm gels. The samples were prepared and the zymogram of alkaline protease activities was obtained as described by García-Carreño et al. (1993) as modified by Alarcón et al. (1998).

2.7. *Statistical analysis*

All data of growth, biochemical composition and intestinal enzyme activities are presented as means ± SEM. The Pearson coeficient of variation (CV) was studied at 23, 56, 87 and 119 dah, with the formula: (treatment standard deviation/treatment mean) × 100 (Sokal and Rohlf, 1981) to determine inter-individual length variation within the same treatment. After testing for normality (Kolmogorov-Smirnof test), a one-way ANOVA was performed to detect statistically significant differences in growth, biochemical composition and enzymatic activities between treatments ($P < 0.05$). In those cases where significant differences were found, a Tukey and Games-Howell post-hoc comparison test (at $\alpha = 0.05$) was applied. Data identified as non-homogeneous (Levene´s test) were normalized appropriately prior to analysis. For data without homogeneous variances after normalization, a more conservative post-hoc test was considered. All statistical analyses were performed using the SPSS v 21 software.

For digestive microbiota studies the DGGE banding patterns obtained were analyzed using FPQuest Software version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated using the Pearson coefficient which considers the location of the bands and their intensities. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method

using Arithmetic Averages (UPGMA). The Pearson similitude coefficients obtained for each treatment were compared using Multiple Range Tests for similarity by diet.

A principal component factor analysis (PCA) was applied to correlate the variables determining growth, body composition, enzyme activity and intestinal microbiota species of sole fish at early stages of development. Kaiser-Meyer-Olkin measures of sampling adequacy and Bartlett's Test of Sphericity (testing the null hypothesis where the original correlation matrix is an identity matrix) were computed for PCA in order to test the validity of the data set. Cumulative proportion of variance criterion was employed in determining the number of factors to extract. The varimax criterion of the orthogonal rotation method was employed for the rotation of the factor matrix. The software used for all these analyses was IBM SPSS Statistics V.20.

3. Results

Growth of larvae (measured as total length and dry weight of fish) was significantly higher after *S. putrefaciens* Pdp11 administration from 10 to 30 dah, as shown in Fig. 1. The probiotic bioencapsulation in *Artemia* metanauplii did not affect survival (88.05-89.83%), but reduced sole growth heterogeneity (Table 1).

Protein and lipid contents of live prey utilized in the feeding trial are summarized in Table 2 where a trend for increasing total protein and lipid content in *Artemia* supplemented with *S. putrefaciens* Pdp11 was observed. Specifically, lipids were higher ($P < 0.05$) in *Artemia* supplemented with *S. putrefaciens* Pdp11- compared to Control *Artemia* (20.5 vs 17.3%). With regard to larval body composition, a clear increment of larval individual nutrients content with age was found after *S. putrefaciens* Pdp11 treatment. For instance, at day 56 after hatching, Pdp11 larvae contained 25.08 mg protein $larva^{-1}$ compared with 20.05 mg $larva^{-1}$ of Control fish. Similarly, at 87 dah Pdp11 group was also significantly richer in fat content (13.56 vs 10.10 mg $larva^{-1}$ for probiotic and Control, respectively) (Fig. 2).

The PCR-DGGE clusters corresponding to sole digestive microbiota at the different sampling days are presented in Fig. 3. A band corresponding to *S. putrefaciens* Pdp11 was sequenced from 23 to 119 dah only in probiotic fish, representing an average intensity of 12.5%, 8%, 5% and 4%, respectively of total intensity showed by all bands of PCR-DGGE patterns. There was a significant gut microbiota modulation in specimens fed *S. putrefaciens* Pdp11-supplemented *Artemia*

compared with those fed Control *Artemia*, this effect being especially consistent at 56 and 87 dah. In this way, PCR-DGGE patterns were clustered in two clear groups, one including control fish (similarity percentage about 40%) and the other corresponding to specimens fed the probiotic diet (similarity percentage about 50%).

S. putrefaciens Pdp11 strain displayed positive for five arylamidase activities (alanyl-phenylalanyl-proline, glutamil β-naftilamide, L-proline, tyrosine and glutamyl–glycyl –arginine). Lipase, N-acetil β-glucosaminidase, phosphatase, succinate alcalinization and Ellman were also positive (Table 3).

Regarding sole larvae, specific and individual enzyme activities showed the same pattern in both experimental groups, with statistical differences ($P < 0.05$) when values were compared among different ages (Fig. 4). However, no difference in the general developmental pattern was observed between both treatments. One way, specific activities (U mg protein^{-1}) progressively increased from 23 to 56 dah, reaching a peak at day 56, and then gradually decreased in older fish. In the other, individual activities (U larva^{-1}) showed a profile characterized by a gradual increase with larval age reaching the maximum value at 119 dah.

When comparing enzyme activities for each sampling day, it was found at day 30 that specific alkaline protease and chymotrypsin activities were significantly higher in larvae fed *Artemia* supplemented with *S. putrefaciens* Pdp11 than fish fed Control *Artemia* (Figs. 4a and 4c). However, when weaning started (56 dah) no difference was found between both experimental groups. At day 56, mean values of specific trypsin activity were higher in Pdp11- group but they were not statistically different from Control group (Fig. 4b). Trypsin and chymotrypsin individual activities of larvae fed *Artemia* supplemented with *S. putrefaciens* Pdp11- were higher than those of Control group, but without a statistically significant difference ($P > 0.05$). α-Amylase, leucine aminopeptidase and alkaline phosphatase activities were not influenced by the addition of *S. putrefaciens* Pdp-11 (Figs. 4d, e and f). After weaning (87 dah), specific activity of α-amylase and alkaline phosphatase in both experimental groups increased, reaching similar values to 56 dah larvae.

The zymogram of alkaline proteases showed the same pattern of active fractions in both experimental groups (Fig. 5). Evidence of the disappearance of the two active bands with the lowest molecular masses at 87 dah was apparent. The remaining active fractions were similar to those detected in *S. senegalensis* juveniles (30 g body weight).

The varimax results of the PCA evaluating the relationship among several are shown in Fig. 6. The three main axes accounted for a total variance of 80.32-91.04% (Table 4). They vary along sole

larviculture (23, 56, 87 and 119 dah) but clearly separate fish of both experimental treatments. The first principal axis compiled between 52.78 and 65.95% of the variance, depending on the sole age. It was related to some intestinal microbiota linked to *S. putrefaciens* Pdp11, and a subsequent enhanced chymotrypsin activity. These *S. putrefaciens* Pdp11 associated microorganisms were not detected in Control fish nor were they present in low concentrations, showing also a lower level of the mentioned proteolytic enzymes (Table 5). The second and third axes of the PCA analysis are linked to other microorganisms and enzyme activities not associated with *S. putrefaciens* Pdp11 administration. They contributed to variance with 13.59-20.04% and 9.29-11.97%, respectively.

4. Discussion

Probiotics have been reported as fish larvae growth promoters in *Solea solea* (Avella et al., 2011) and *Epinephelus coioides* (Sun et al., 2013). In this way a previous study with *Shewanella putrefaciens* Pdp11 supplied in a longer pulse (10-86 dah) improved final growth in *Solea senegalensis* larvae (Lobo et al., 2013). The present study demonstrated that a short probiotic pulse (10-30 dah) is enough to significantly improve growth performance of sole during their ontogenetic development. The positive nutritional effect detected during early stages might persist along the trial as it has been described in Atlantic halibut *Hippoglossus hippoglossus* (Naess and Lie, 1998) and other flatfish species (Dámaso-Rodrigues et al., 2010). Growth obtained in the present study was higher than those registered by Cañavate and Fernández-Díaz (1999) and Ribeiro et al. (2005) in sole postlarvae, but slightly lower than those reported by Engrola et al. (2007), which might be due to the higher rearing temperature utilized by these authors (21 ºC).

Enhancing larval development is a relevant characteristic of probiotic utilization in fish aquaculture (Avella et al., 2010). The significantly higher length detected after the end of metamorphosis for Pdp11 larvae has been previously linked to an advanced metamorphosis (Lobo et al., 2013). Similar findings have been reported in *Perca fluviatilis* larvae with a mix of three *Bacillus* sp. (Mandiki et al., 2011). In addition a short pulse of *S. putrefaciens* Pdp11 might contribute to producing not only an advanced, but a more synchronized metamorphosis in sole larviculture.

The size dispersion obtained in the present study was lower than that described in other flatfish species (Lee et al., 2000) including *S. senegalensis* (Engrola et al., 2005, 2007). In this sense a short

pulse of *S. putrefaciens* Pdp11 contributes to reducing handling activities and labour costs related to size grading in sole culture, with the consequent advantage for future juvenile performance.

S. putrefaciens Pdp11 administration did not enhance survival as described for other probiotics applied to farmed fish (Tinh et al., 2008). Likewise, our survival values were higher than those reported by other authors for the same species (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2005; Lobo et al., 2013).

Fish fed *S. putrefaciens* Pdp11 *Artemia* showed higher levels of intestinal proteolytic activities than Control fish up to weaning. This effect has been also detected in sole juveniles fed *S. putrefaciens* Pdp11 supplied in feed (Sáenz de Rodrigáñez et al., 2009). A stimulation of digestive enzyme activity after the use of different probiotics in fish culture was also described (Suzer et al., 2008). The higher proteolytic activity level in Pdp11 group close to weaning might be related to the higher growth observed as a consequence of a more efficient inert diet utilization, as has been reported by probiotic administration in *A. Persicus* (Askarian et al., 2011) and *Ctenopharyngodon idella* (Wu et al., 2012), respectively. The replacement of live prey by inert diets frequently produces a reduction in sole postlarvae growth rates linked to the new feed digestion (Ribeiro et al., 2005; Engrola et al., 2007) as is observed in this study. Results obtained show a progressive increase in larval enzyme activity, together with a progressive decrease in the specific activity of almost all the digestive enzymes after day 56. Since both experimental groups showed the same pattern of variation, it can be deduced that these changes are genetically programmed as a consequence of fish development (Henning, 1987). A zymogram of alkaline protease activity in *S. senegalensis* larvae showed the same profile of active fractions in both groups through experimental period. Active isoforms with the lowest molecular masses visualized at days 23, 30 and 56 turn off after completion of weaning (day 87). This result matches with the above mentioned decrease in specific activity levels observed close to the weaning period. At 87 dah the zymogram is similar to those detected in juvenile specimens, which confirms the end of the maturation process of intestinal proteases in this species. Results confirmed that a short pulse of *Artemia* supplemented with *S. putrefaciens* Pdp11 throughout *S. senegalensis* larviculture increases the amount but does not modify the composition of intestinal proteases secreted into the intestinal lumen. Thus the inclusion of additives, such as *S. putrefaciens* Pdp11, to improve nutrient digestion of inert diets will be of interest for the sole industry in the future.

Fish larvae are characterized by an extremely rapid growth coupled with high demands for energy and structural components (Sargent et al., 2002). Lipid levels and protein/lipid ratio in Control *Artemia* used in the present study were similar to those of sole hatcheries (Morais et al., 2006; Boglino et al., 2012). Moreover, the chemical inert diet composition utilized in this trial was similar to that usually administered in *S. senegalensis* facilities (Rema et al., 2008). Proteolytic activities detected in *S. putrefaciens* Pdp11 strain might be responsible for the higher protein digestion and absorption observed in both *Artemia* supplemented with *S. putrefaciens* Pdp11 and corresponding fed larvae leading to a subsequent better larval growth and advanced metamorphosis. In relation to the significantly higher lipid levels of *Artemia* supplemented with probiotic metamorphosis might be also facilitated by this initial energetic input, the corresponding protein sparing effect (Rodríguez et al., 2009; Li et al., 2012) and a consequent advanced and sustained growth. Lipase activity detetcted in *S. putrefaciens* Pdp11 strain and probably that of sole larvae, stimulated by the higher lipid level of *Artemia* supplemented with *S. putrefaciens* Pdp11, enhanced fish metabolism and growth. In this sense an increase of lipase activity and growth has been reported when lactic acid bacteria were supplied to Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) (Askarian et al., 2011). Both protease and lipase activities detected in *S. putrefaciens* Pdp11 strain might be considered an interesting characteristic for probiotic uses. In this context an increase in proteolytic activities and an improvement of the nutritive condition of *Artemia* related to probiotic digestion linked to a microbiota modulation has been reported (Verschuere et al., 1999; Ahmadnia-Motladh et al., 2012).

S. putrefaciens Pdp11 highly modulated larval and fry gut microbiota as has also been reported in sole juveniles (Tapia-Paniagua et al., 2012). Microbiota modulation by oral probiotic bacteria has also been reported in turbot (*S. maximus*) (Ringo, 1999), rainbow trout (*Oncorhynchus mykiss*) (Robertson et al., 2000) and grouper (*E. coioides*) (Sun et al., 2013). The DGGE patterns obtained from the intestinal microbiota of 56 and 87 dah postlarvae showed a clear trend to cluster the fish fed probiotic diet, whereas this trend was less accentuated at 23 and 119 dah. Our results might indicate that a short pulse of *S. putrefaciens* Pdp11 produces a steering effect towards a stabilization of the intestinal microbiota of sole larvae, especially at weaning period (56 to 87 dah). Our findings are in agreement with that observed by De Schryver et al. (2010), who also reported a requirement of a transient time to establish functional microbial community in juvenile seabass (*D. labrax*) fed with a diet supplemented with poly-β-hydroxibutyrate (PHB). Likewise a lower band pattern similarity was observed at 119 dah that might indicate a lower *S. putrefaciens* Pdp11 effect at this

age, three months after probiotic administration. However a band corresponding to *S. putrefaciens* Pdp11 was sequenced from 23 to 119 dah only in Pdp11 fish. This fact confirms the capability of long- term gut colonization for this probiotic strain reported in a previous study (Lobo et al., 2014). This gut colonization ability at first stages of *S. senegalensis (*23 dah) culture when digestive tract is not fully developed (Ribeiro et al., 1999; Padrós et al., 2011) seems relevant in relation to immune and nutritive condition of specimens. In this context similar gut colonization competence has been previously described for several probiotic species supplied to farmed fish (Tinh et al., 2008; Sun et al., 2013). The changes observed in the intestinal microbiota at 56 and 87 dah may also be related with the increase of weight and length of sole detected in our study. A similar relationship between intestinal microbiota and growth of fish fed probiotics and prebiotics have been reported in seabass (D. labrax) and grouper (E. coioides) by several authors (Carnevali et al., 2006; De Schryver et al., 2010).

A diversified microbiota was observed in the digestive system of sole larvae and fry along the culture after sequencing of larval extracted DNA. This fact was related with both diets (live and inert) and sole developmental stage. Moreover probiotic supply of *S. putrefaciens* Pdp11 strongly determined specific gut microbiota composition in relation to Control. Microbiota modulation may be a beneficial characteristic for fish gut avoiding the presence of opportunistic and pathogen bacteria (Yang et al., 2012) and developing an enhanced immune response (Taoka et al., 2006; Picchietti et al., 2007). In this context, it is worthpointing out the absence or lower frequency of the pathogenic bacteria *Photobacterium damselae* subps *piscicida* (Zarza and Padrós, 2008) in fish fed *S. putrefaciens* Pdp11. Moreover it is interesting to emphasize the presence of probiotic strains like *Lactobacillus helveticus* in the gut of *S. putrefaciens* Pdp11 fish. A more detailed gut microbiota study has been carried out to compare the relationship between diets (Control and probiotic) and intestinal bacteria along sole first developmental stages.

A PCA analysis, including all the parameters analyzed, strongly differentiated fish fed with probiotic from Control fish. There was a microbiota selection carried out by live feed supplemented with *S. putrefaciens* Pdp11 linked to an enhanced enzymatic activity. Both conditions resulted in a better growth performance along sole larval and fry culture.

5. Conclusion

In conclusion a short pulse of *S. putrefaciens* Pdp11 (10-30 dah) seems sufficient to obtain a suitable microbial modulation involved in digestive enzyme stimulation and growth and body composition enhancement. Moreover size variability was smaller from metamorphosis until the end of weaning for probiotic fish. Both the enhancement of the nutritive condition and the lower size variability observed in fish related to *Artemia* supplemented with *S. putrefaciens* Pdp11 diet hold great potential for sole aquaculture These results emphasize the need to develop tailor-made probiotic solutions for successful larviculture.

Conflict of interest

This research does not present conflicts of interest.

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Table 3. Enzymatic profile potential of the probiotic *Shewanella putrefaciens* Pdp11 deduced from the ID-GNB Vitek 2 (Biomerieux, Marcy L'Etoile, France) test.

Enzyme activity	Reaction catalyzed/physiological function	Quantity (mg)
Ala-Phe-Pro-arylamidase	Exoprotease that facilitates the liberation of peptides with Ala-Phe-Pro terminal sequence from proteins.	0.0384
B-N-acetil-glucoaminidase (Chitinase)	Enzyme that liberates N-acetilglucosamine	0.0408
Glutamil arylamidase pNA	Exoprotease that liberates peptides with Glu terminal from proteins	0.0324
L-Proline-arylamidase	Exoprotease that liberates peptides with Pro terminal from proteins	0.0234
Tyrosine arylamidase	Exoprotease that liberates peptides with Tyr terminal from proteins	0.0276
Glu-Gly-Arg-arylamidase	Exoprotease that facilitates the liberation of peptides with Glu-Gly-Arg terminal sequence from proteins.	0.0376
Lipase	Activity involved in lipid digestion hydrolyzing esters of glycerol with preferably long chain fatty acids	0.0192
Phosphatase	Enzyme involved in nutrition and mineralization processes increasing phosphorus bioavailability	0.0504
Succinate alcalinization	Activity relieving acidic stress from aminoacid metabolism	0.15
Ellman	It indicates the presence of free thiols in the probiotic strain. It confers protein stability and protection against oxidative stress	0.03

Table(s)

Table 4 Variance accounted for the PCA axes of Senegalese sole larval and fry parameters (23, 56, 87 and 119 dah)

	Components			
	1	2	3	
Culture day	% variance			Total
23 dah	64.1	15.3	9.29	88.7
56 dah	58.8	20.0	12.0	90.8
87 dah	66.0	13.6	10.5	90.0
119 dah	52.8	15.7	11.5	80.0

Table(s)

Table 1. Survival and size variability of Senegalese sole specimens fed Control *Artemia* and *S. putrefaciens* Pdp11 supplemented *Artemia* (10-30 dah). Different letters denote significant difference ($P \leq 0.05$) among treatments (mean ± SEM).

	2 DAH		26 DAH		41 DAH		56 DAH		87 DAH		119 DAH	
	Control	**Pdp11**	**Control**	**Pdp11**	**Control**	**Pdp11**	**Control**	**Pdp11**	**Control**	**Pdp11**	**Control**	**Pdp11**
Length dispersion	4.29 ± 0.04	4.29 ± 0.04	4.89 ± 0.31[a]	3.90 ±.0.38 [b]	4.77 ± 0.73	4.76 ± 0.29	8.30 ± 0.38[a]	7.54 ± 0.33[b]	12.9 ± 0.4[a]	8.64 ± 1.57[b]	15.6 ± 1.5[a]	13.6 ± 1.4[b]
Survival %	----	----	97.8 ± 0.2	97.1 ± 0.8	97.5 ± 0.3	96.7 ± 0.9	97.4 ± 0.2	96.6 ± 1.0	97.0 ± 0.1	96.3 ± 1.0	89.8 ± 0.7	88.1 ± 1.3

Table(s)

Table 2. Protein and lipid content (g 100^{-1} g dry weight) of Control and *S. putrefaciens* Pdp11 supplemented *Artemia* (mean ± SEM). Different lowercase letter denotes significant differences ($P \leq 0.05$) between treatments.

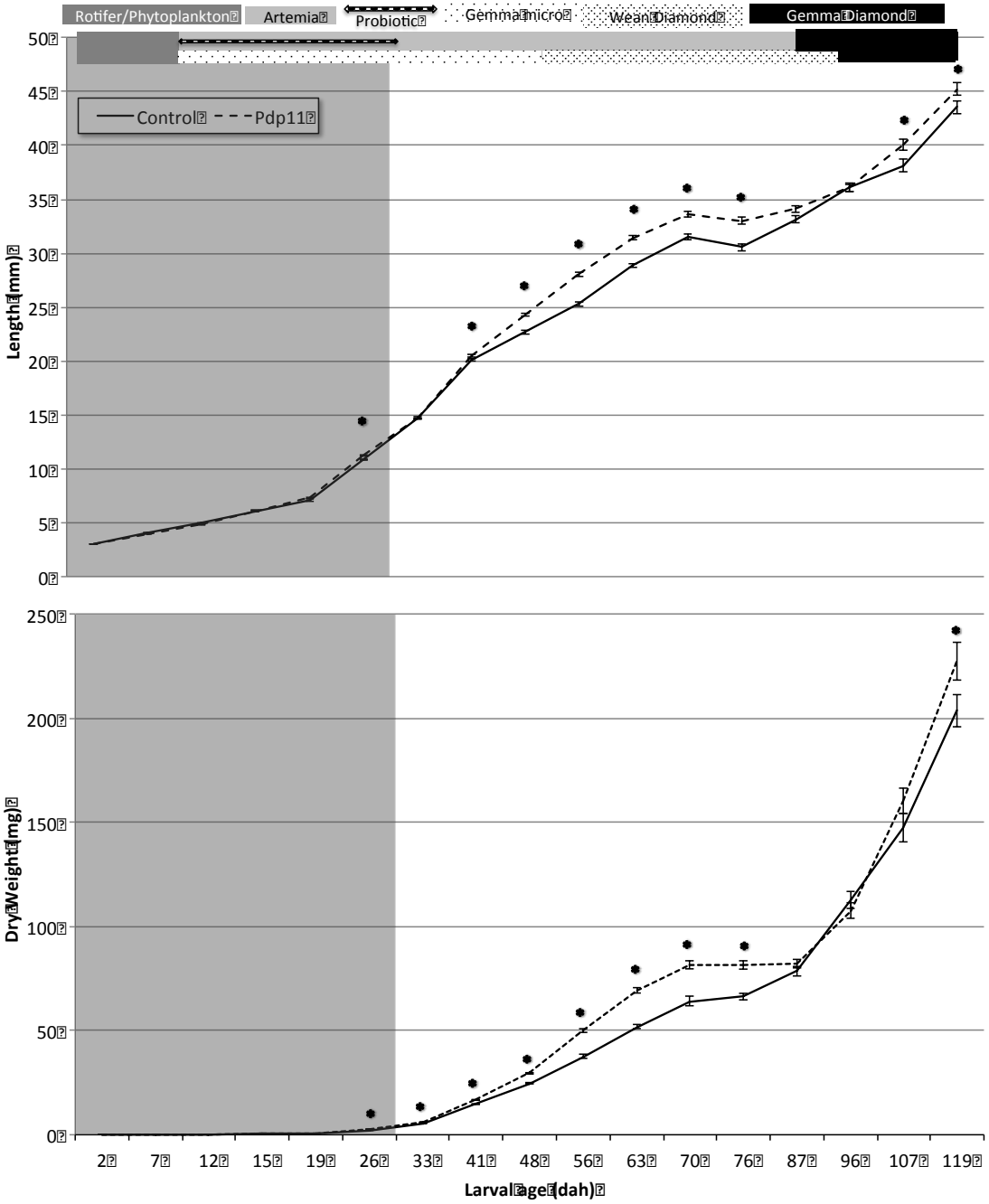
	Live prey	
	Control *Artemia*	**Pdp11 *Artemia***
Crude protein	36.4 ± 0.3	40.5 ± 1.1
Crude lipid	17.3 ± 0.2[a]	20.5 ± 0.2[b]
Protein/lipid rate	2.11 ± 0.00	1.98 ± 0.04

Table(s)

Table 5. Eigeenvalues and loading factor after varimax rotation of sole larvae and fry fed Control *Artemia* and *S. putrefaciens* Pdp11supplemented *Artemia* at 23, 56, 87 and 119 dah. Bold letters indicate loading factors higher than 0.80.

	23 dah			56 dah			87 dah			119 dah		
Component	1	2	3	1	2	3	1	2	3	1	2	3
Variable												
Length	**0,98**	-0,14	0,00	**1,00**	0,05	-0,04	**0,99**	0,05	-0,10	**-0,94**	0,14	0,26
Dry Weight	**0,96**	-0,25	-0,10	**1,00**	0,06	-0,04	**0,98**	0,06	-0,12	**-0,91**	0,04	0,25
Protein %	-0,76	-0,52	-0,09	**-0,80**	0,13	-0,32	-0,43	0,00	**0,82**	0,27	0,03	-0,74
mg Proteín/1 Larva	0,29	**-0,83**	-0,03	**0,94**	0,20	-0,19	-0,18	0,01	**0,88**	-0,23	0,07	-0,79
Lipid %	**0,89**	-0,25	0,31	-0,18	0,14	0,03	**0,91**	0,06	0,15	0,50	-0,29	0,69
mg Lípid/1 Larva	**0,96**	-0,18	0,18	**0,90**	0,15	-0,05	**0,94**	0,07	0,11	-0,05	-0,31	**0,82**
T_Protein/Lípid	**-0,92**	-0,23	-0,21	-0,42	-0,02	-0,25	**-0,88**	0,00	0,21	-0,16	0,18	**-0,92**
T_mU/mg protein	0,14	**0,87**	0,12	0,15	**0,95**	0,21	0,05	**0,90**	0,16	-0,34	0,68	0,22
T-mU/larva	0,11	0,71	-0,21	0,15	**0,94**	0,30	0,08	**0,88**	-0,46	-0,12	**0,83**	-0,06
Q-mU/mg protein	**0,85**	0,34	0,21	0,10	**0,98**	-0,04	0,19	**0,88**	0,32	-0,12	**0,86**	0,16
Q-mU/larva	**0,91**	0,18	0,09	0,18	**0,98**	0,06	0,19	**0,92**	-0,33	-0,10	**0,92**	0,07
A-mU/mg protein	0,39	0,47	**0,69**	-0,18	**0,96**	-0,14	-0,03	0,41	**-0,79**	0,26	0,65	-0,35
A-mU/larva	-0,23	0,69	**0,63**	-0,10	**0,86**	-0,07	0,01	0,62	**-0,74**	0,12	**0,87**	-0,41
FA-mU/mg protein	-0,02	**0,90**	0,43	0,05	0,71	0,60	0,49	0,28	0,31	-0,42	0,57	0,31
FA-mU/larva	-0,31	-0,23	**-0,85**	0,15	0,55	0,78	0,15	0,73	-0,45	-0,09	**0,87**	-0,12
LA-mU/mg protein	-0,28	0,76	0,45	-0,06	-0,17	**0,92**	-0,17	-0,05	0,30	-0,48	0,22	0,35
LA-mU/larva	-0,57	0,45	0,14	0,06	-0,30	**0,94**	0,06	0,69	-0,67	-0,02	0,74	-0,27
PA-mU/mg protein	0,68	-0,33	**0,66**	0,00	**0,98**	0,02	-0,17	**0,93**	0,13	-0,14	0,76	0,35
PA-mU/larva	0,63	-0,71	0,32	0,10	**0,97**	0,15	-0,07	**0,91**	-0,39	-0,02	**0,94**	0,11
Pseudomonas	**-0,99**	0,12	-0,07	**1,00**	0,07	-0,03				0,50	0,36	0,14
Marine bacterium B37	**-0,99**	0,12	-0,07	**-1,00**	-0,07	0,03				**1,00**	-0,06	0,06
Enterobacter sp 11H	0,24	0,46	-0,80	**-1,00**	-0,07	0,03				**1,00**	-0,06	0,06
Halomonas sp AMP12	**-0,99**	0,12	-0,07	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Acinetobacter sp MBL 11-0202	**0,99**	-0,12	0,07	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	**0,83**	0,09	-0,03
Brevibacillus paravebris DYJK58	**0,99**	-0,12	0,07	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Mycoplasma microti L371	**0,99**	-0,12	0,07	**-1,00**	-0,07	0,03				**1,00**	-0,06	0,06
Rhodococcus erythropolis H11	-0,35	**0,80**	-0,17				**0,99**	0,05	-0,10	0,73	-0,23	0,05
Uncultured Mycoplasma sp Kb.1	**-0,99**	0,12	-0,07				**0,99**	0,05	-0,10	**-0,82**	-0,01	-0,14
Pseudomonas sp FG-12b				**1,00**	0,07	-0,03	-0,77	0,36	0,43	0,65	-0,10	-0,16
Acinetobacter sp CmNA3	**-0,99**	0,12	-0,07	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	**-1,00**	0,06	-0,06
Enterobacter sp CC-SN15-5	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	0,08	-0,01	0,77	**1,00**	-0,06	0,06
Bacterium A119	**0,99**	-0,12	0,07	0,41	0,73	-0,29	**-0,99**	-0,05	0,10	-0,67	-0,17	-0,06
Geobacillus sp V1W70BlkII	0,55	0,38	-0,70	**-1,00**	-0,07	0,03	-0,73	-0,48	0,21	-0,64	-0,23	-0,19
Rhodococcus sp TM1	**-0,99**	0,12	-0,07	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Klebsiella sp PG4-2	-0,35	**0,80**	-0,17	0,31	-0,30	0,50	-0,77	0,36	0,43	**1,00**	-0,06	0,06
Anoxybacillus sp D503				-0,74	0,36	0,32	**-0,99**	-0,05	0,10	-0,08	-0,59	0,31
Vibrio sp DMS01				-0,43	0,20	**0,85**	**-0,99**	-0,05	0,10	-0,08	-0,59	0,31
Vibrio campbelli HNS034	**-0,99**	0,12	-0,07	-0,74	0,36	0,32	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Photobacterium damselae subsp piscicida pRDA19	**-0,99**	0,12	-0,07	-0,43	0,20	**0,85**	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Vibrio natriegens D1/252	**-0,99**	0,12	-0,07				0,59	0,33	0,14	**-0,96**	-0,07	-0,10
Allivibrio sp SW5-1	**-0,99**	0,12	-0,07	-0,10	0,25	**0,93**	**0,99**	0,05	-0,10	0,29	-0,09	**0,82**
Uncultured Anoxybacillus sp K.b-8	**-0,99**	0,12	-0,07				**-0,99**	-0,05	0,10			
Candidadtus arthromitus sp SFB-rat-Yit	-0,43	-0,27	-0,63	-0,73	0,50	-0,20	0,62	0,07	0,29	**-1,00**	0,06	-0,06
Candidadtus arthromitus sp SFB-rat-Yit	**-0,99**	0,12	-0,07							-0,77	0,28	-0,37
Pseudomonas sp Iso-13	0,66	-0,43	-0,10	**-1,00**	-0,07	0,03	**0,99**	0,05	-0,10	**0,93**	-0,16	-0,01
Bacterium PJ-35							**0,99**	0,05	-0,10	**0,89**	-0,18	-0,02
Pseudomonas sp Ant-4	-0,36	0,00	0,09	**-1,00**	-0,07	0,03	**-0,99**	-0,05	0,10	-0,03	0,37	0,68
Uncultured bacterium R4J7L4_B3	0,42	-0,12	0,28	0,41	0,73	-0,29	**-0,99**	-0,05	0,10	**1,00**	-0,06	0,06
Pseudomonas fluorescens LMG5329	-0,77	-0,51	0,06	**1,00**	0,07	-0,03	**-0,89**	0,11	0,19			
Uncultured bacterium cd89b06c1	0,72	0,21	0,40	**1,00**	0,00	-0,08	**-0,99**	-0,05	0,10	0,76	0,09	0,21
Uncultured bacterium BR01AD06				**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06
Candidatus arthromitus	0,72	0,21	0,40				**0,99**	0,05	-0,10	-0,44	-0,11	-0,73
Lactobacillus helveticus GIMC23:KSN	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06
Pseudomonas cephalitica nd01	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06
Vibrio parahaemolyticus qdfsVp001	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10			
Shewanella putrefaciens pdp11	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06
Shewanella ANA	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06
Vibrio sp Pelg0901	**0,99**	-0,12	0,07	**1,00**	0,07	-0,03	**0,99**	0,05	-0,10	**-1,00**	0,06	-0,06

Figure(s)


Rotifer/Phytoplankton
Artemia
Probiotic
Gemma micro
Weani Diamond
Gemma Diamond
Control
Pdp11
Length (mm)
Dry Weight (mg)
Larval age (dah)
0
5
10
15
20
25
30
35
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45
50
0
50
100
150
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250
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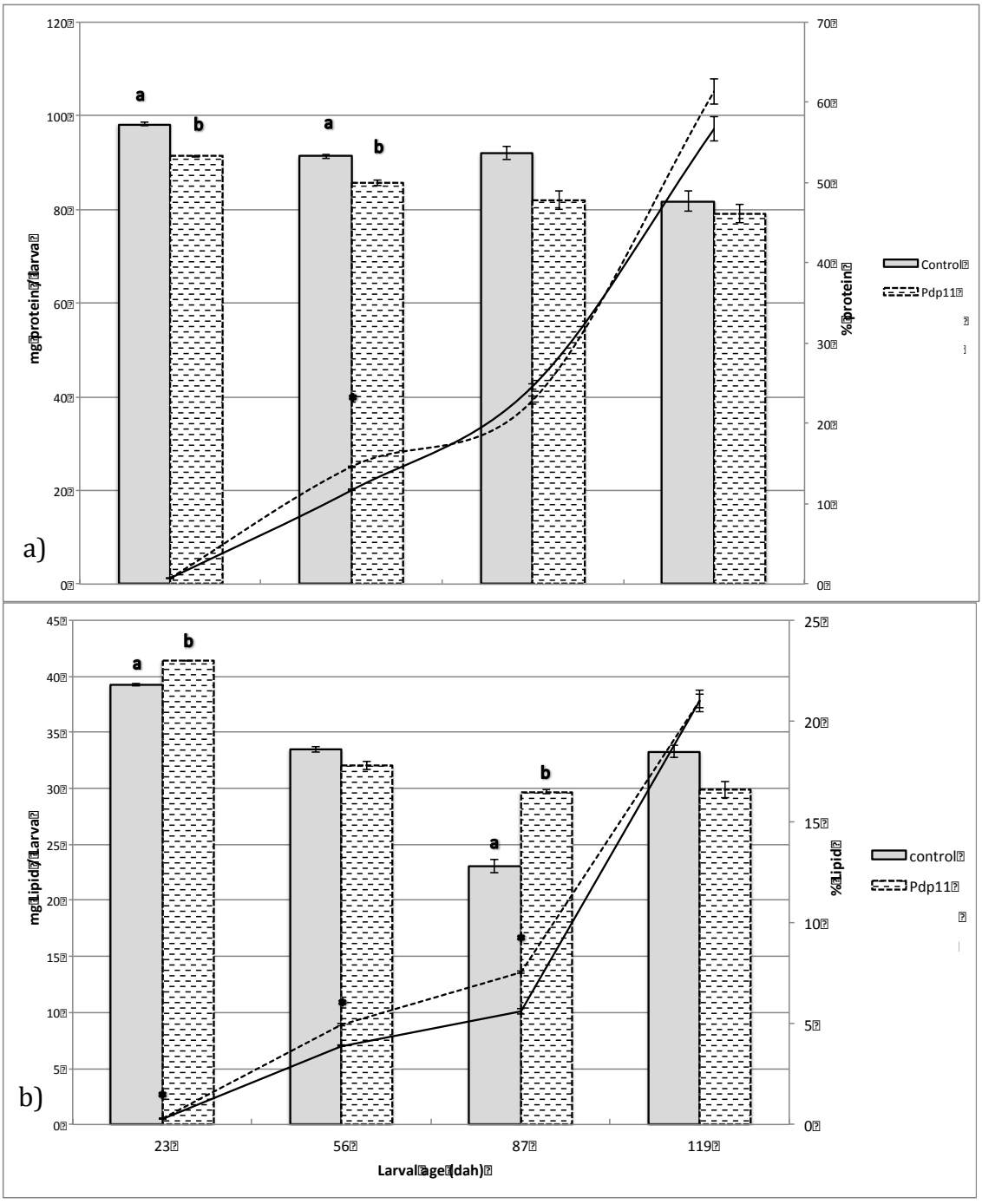
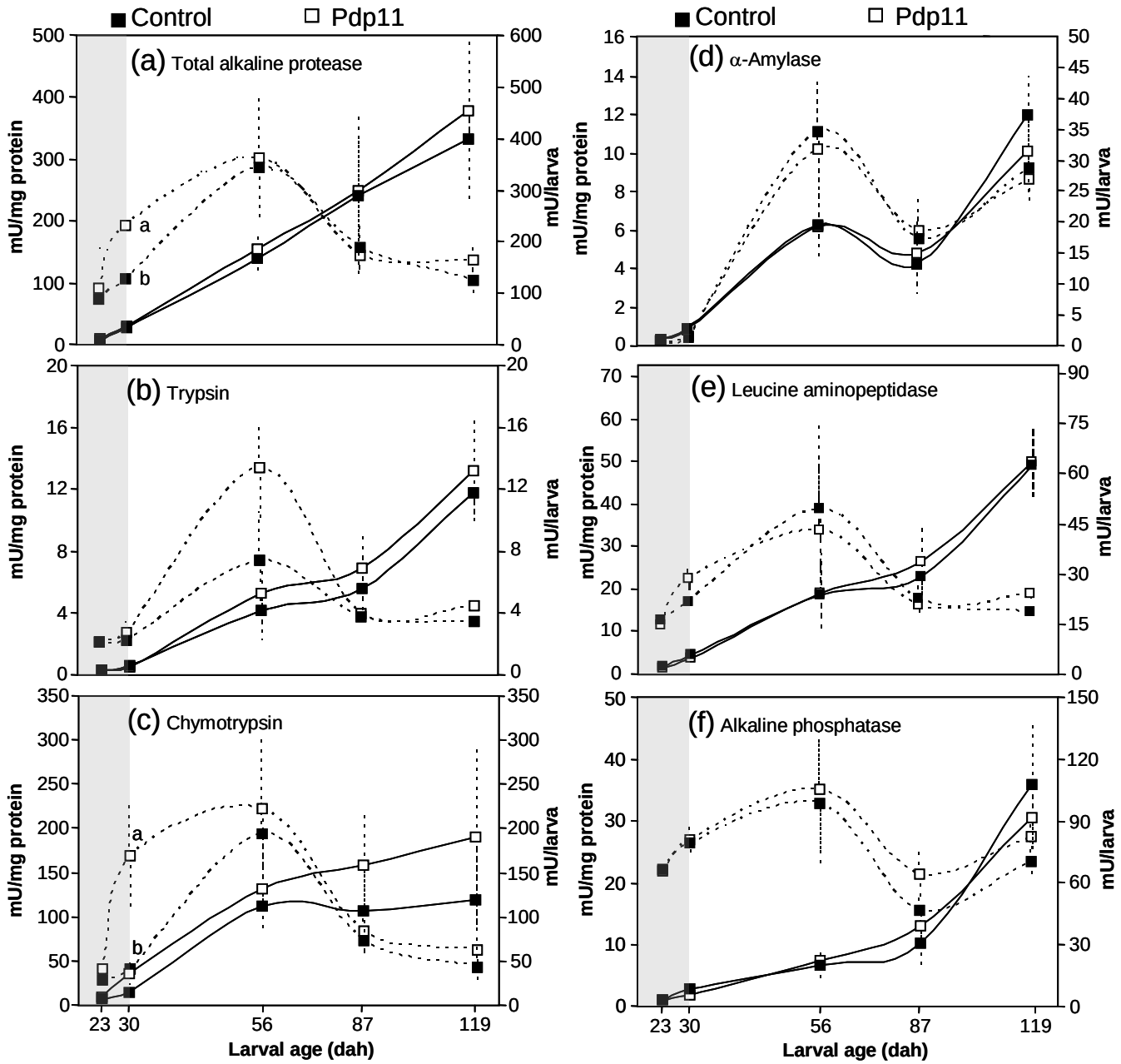
107
119

Figure(s)


a)
mg protein/larva
% protein
Control
Pdp11
a
b
b)
mg Lipid/Larva
% Lipid
control
Pdp11
23
56
87
119
Larval age (dah)

Figure(s)


Control
Pdp11
(a) Total alkaline protease
(b) Trypsin
(c) Chymotrypsin
(d) α-Amylase
(e) Leucine aminopeptidase
(f) Alkaline phosphatase
mU/mg protein
mU/larva
a
b
23 30
56
87
119
Larval age (dah)

Figure(s)

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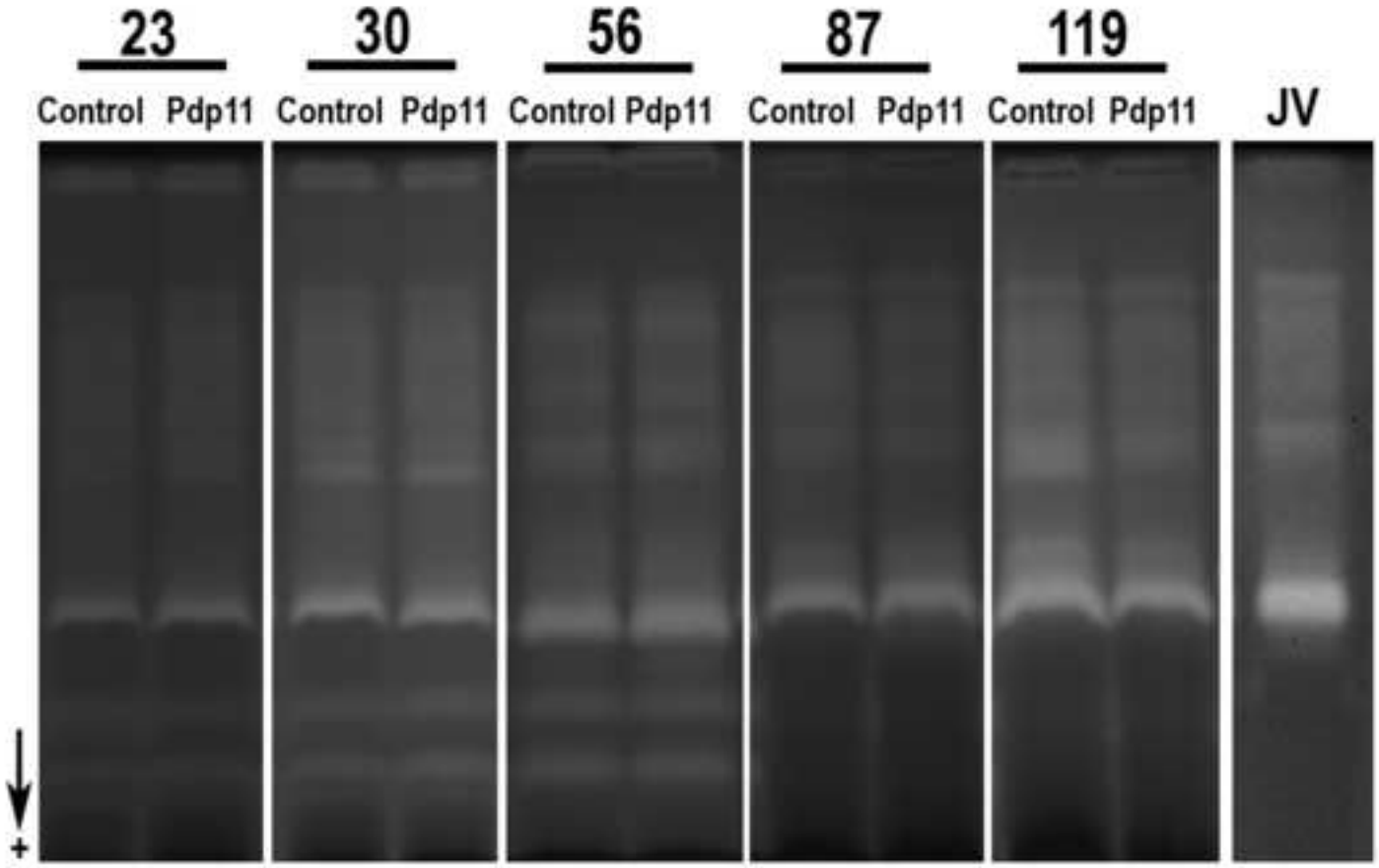
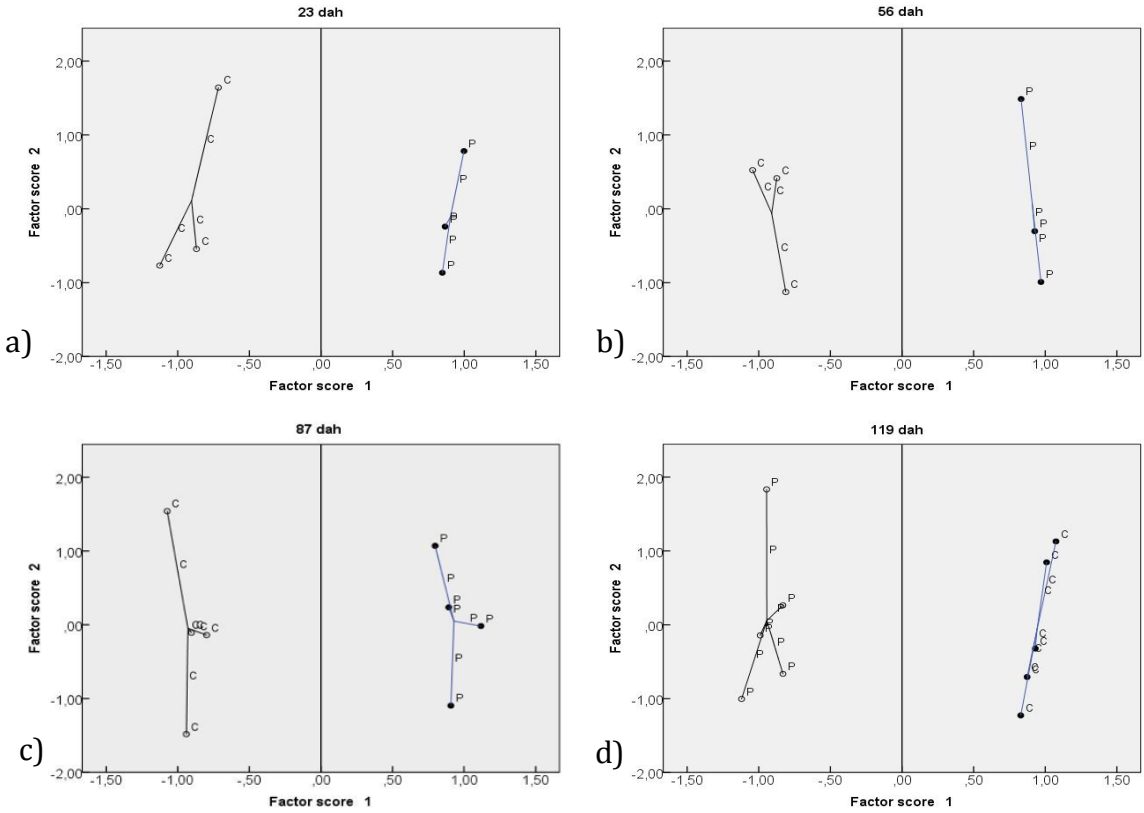


Figure(s)


23 dah
56 dah
87 dah
119 dah
Factor score 2
Factor score 1
2,00
1,00
,00
-1,00
-2,00
-1,50
-1,00
-,50
,00
,50
1,00
1,50
a)
b)
c)
d)

Figure(s)

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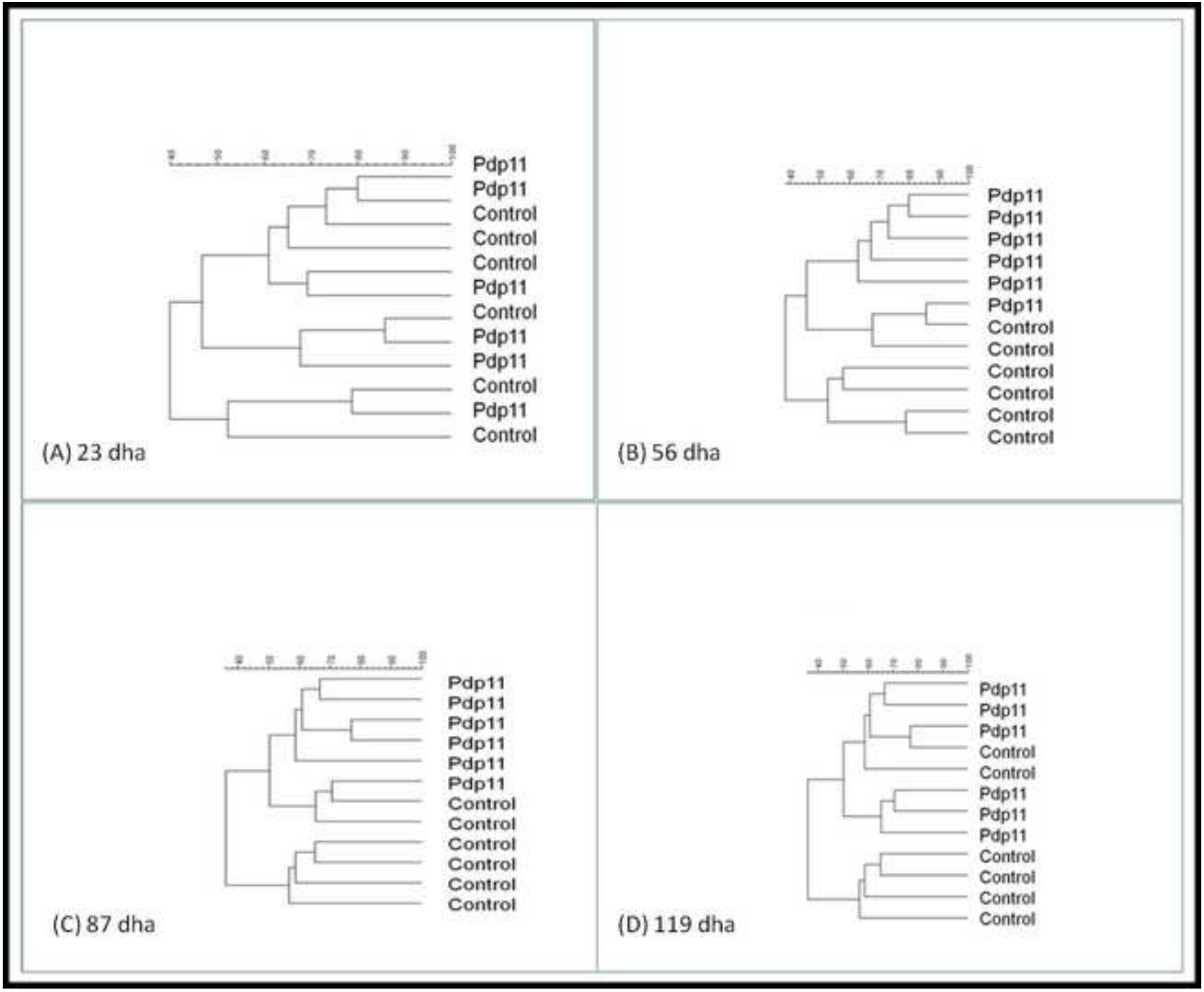

Figure legends:

Fig 1. Feeding protocol and larval growth in (a) total length (mm) and (b) dry weight (mg) of Senegalese sole larvae fed Control *Artemia* and *S. putrefaciens* Pdp11 supplemented *Artemia* (10-30 dah). Values are mean ± SEM of triplicate determination. Asterisk (*) for the same sampling day denotes significant differences between treatments ($P < 0.05$).

Fig. 2. Protein (g x 100 g^{-1} dry weigh (a) and lipid content and mg x $larvae^{-1}$) (b) of Senegalese sole specimens fed Control *Artemia* and *S. putrefaciens* Pdp11 supplemented *Artemia*. Values are mean ± SEM of triplicate determination. Different lowercase letters and asterisks (*) for the same sampling day denote significant differences between treatments ($p \leq 0.05$).

Fig 3. PCR-DGGE clustering computed on similarity Pearson coefficients of intestinal microbiota of Senegalese sole specimens at different sampling days: A) 23, dah, B) 56 dah, C) 89 dah, and D) 119 dah. Experimental treatments are fish fed Control *Artemia* and *S. putrefaciens* Pdp11 supplemented *Artemia*

Fig. 4. Evolution of digestive enzymatic activities (total alkaline protease, trypsin, chymotrypsin, amylase, leucine aminopeptidase and alkaline phosphatase) in gut homogenates of Senegalese sole specimens at 23, 30, 56, 87 and 119 dah**.** Experimental treatments are Control *Artemia* (□) and *S. putrefaciens* Pdp11 supplemented *Artemia* (□) from 10 to 30 dah. Dashed and continuous lines represented specific (U/mg protein) and individual (U/larvae) enzyme activities, respectively. Values are mean ± SEM of triplicate determination. Different lowercase letters for the same sampling day denote significant differences between dietary treatments ($P < 0.05$).

Fig. 5. Zymogram on total proteolytic activity from pooled Senegalese sole larval gut extracts at 23, 30, 56, 87 and 119 dah. For comparative purpose, lane JV shows the pattern of intestinal proteases obtained from a juvenile specimen (30 g).

Fig 6. Effect of Control *Artemia* and *S. putrefaciens* Pdp11supplemented *Artemia* on Senegalese sole culture variables (total length, dry weight, protein and lipid content, digestive enzymatic activities and gut microbiota) defined by the PCA axes (23, 56, 87 and 119 dah).